Figure 1 A

SEQ ID NO: 1

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KNCAE

CDTLVCLSEMRAHIRTCQKYIDKYGPLQELEETAARCVCPFCQRELYEDSLLDHC
ITH

HRSERRPVFCPLCRLIPDENPSSFSGNLIRHLQVSHTLFYDDFIDFNIIEEALIRRVL
DRSLLEYVNHSNTT"

Figure 1B

SEQ ID NO: 2

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SEQ ID NO: 4   Figure 1 D

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```

Figure 1 E

TRAC1 genomic region:

SEQ ID NO: 5

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SEQ NO:5
cont'd

Figure 1 E cont'd

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Figure 1 F

Mouse TRAC1 cDNA sequence:

```
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Mouse TRAC1 protein (3rd frame)

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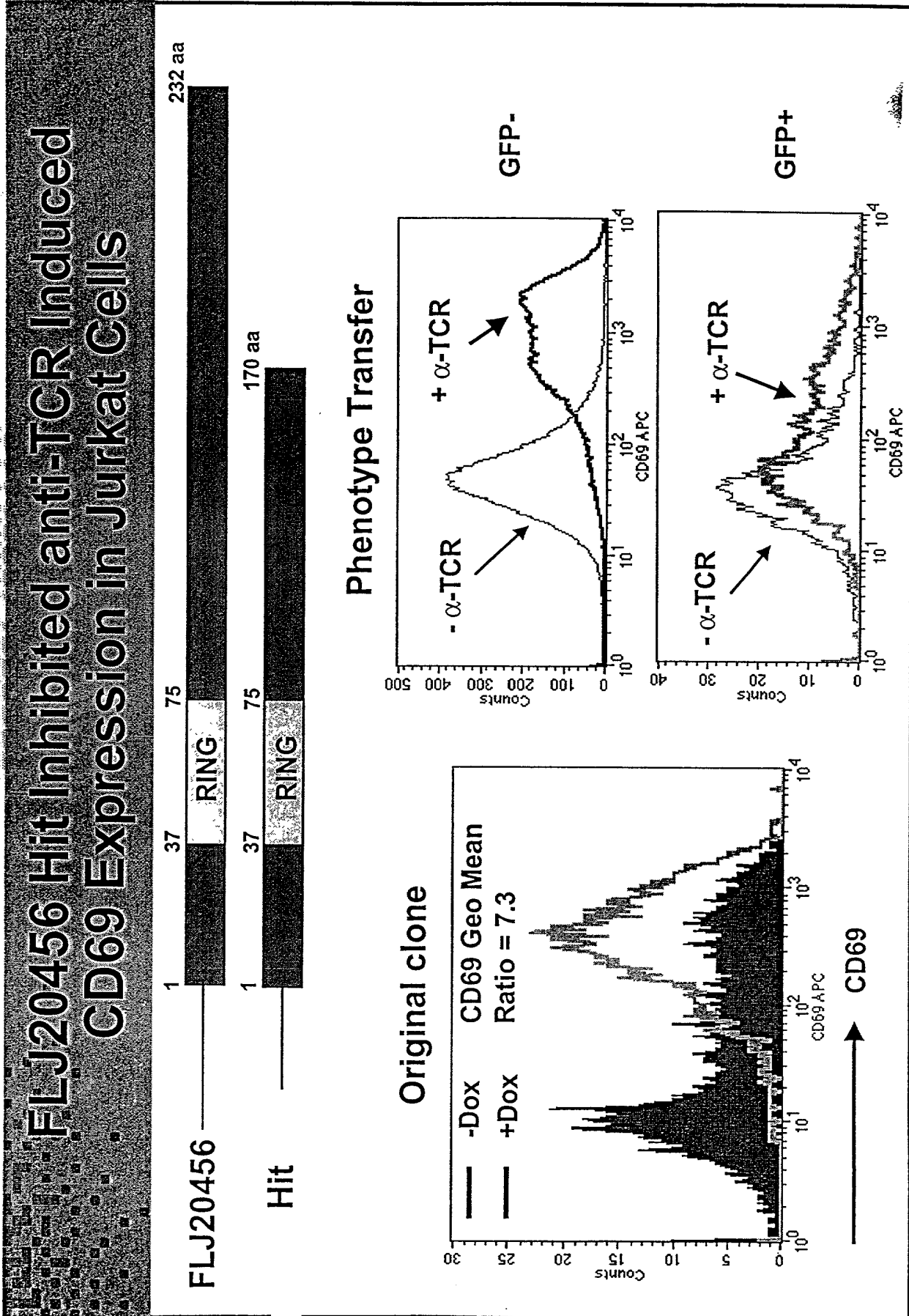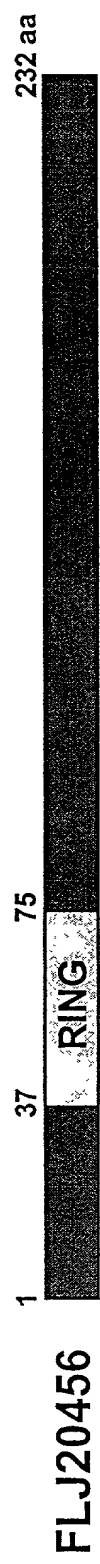
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Figure 2

FLJ20456 Hit Inhibited anti-TCR Induced CD69 Expression in Jurkat Cells

Figure 3

Full Length FLJ20456 Does Not Inhibit CD69 Upregulation in Jurkat Cells

FLJ20456

1   37   RING   75   232 aa

• Pfu PCR product amplified from a capped human brain cDNA library.
• One N to S polymorphism with FLJ20456 NM_017831.1 at amino acid 186, present in EST database.

JurkatN 32H

300ng/ml C305
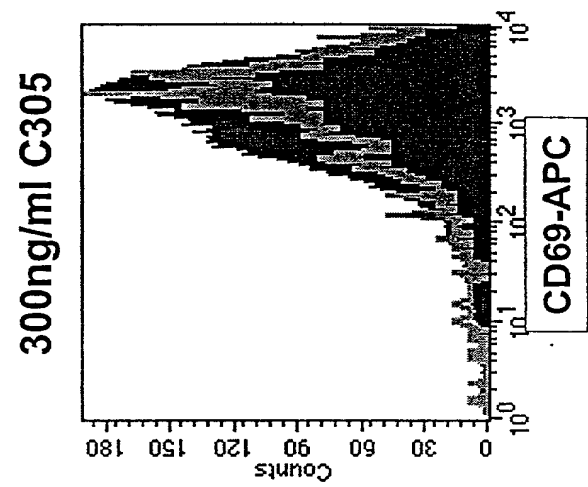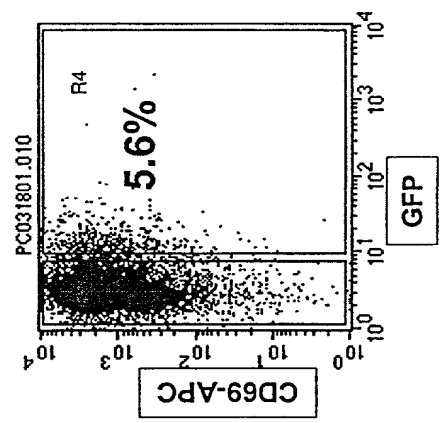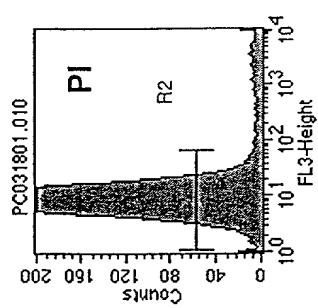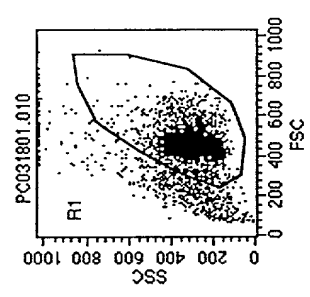
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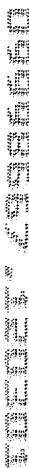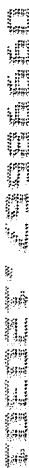
Figure 4

Figure 5

FLJ20456 Is Strongly Expressed in Lymphoid and Hematopoietic Organs

Probe: FLJ20456 Hit

1   37   75   170 aa

RING

FLJ20456

kb

9.5
7.5

4.4

2.4

1.35

0.24

Brain
Heart
Skeletal muscle
Colon
Thymus
Spleen
Kidney
Liver
Small intestine
Placenta
Lung
PBL
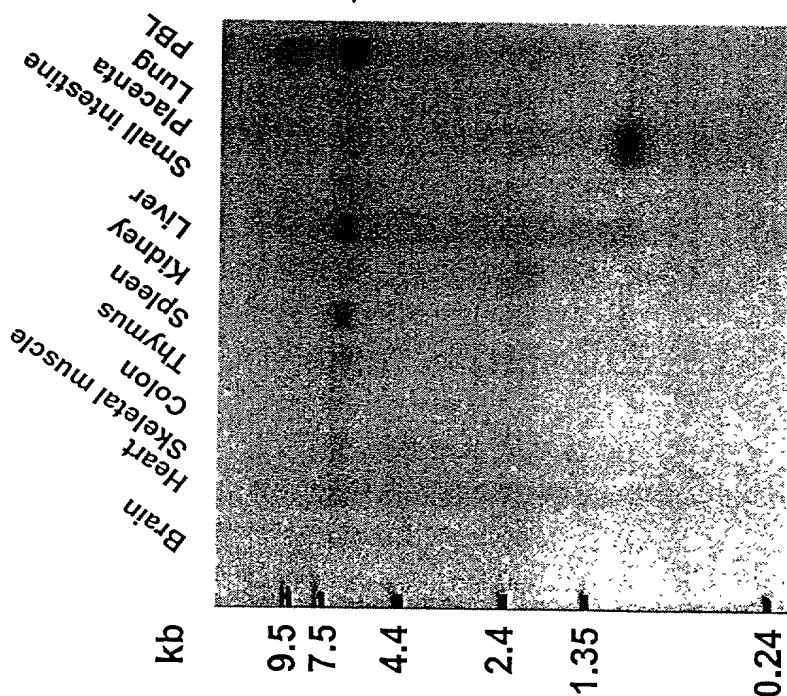
- FLJ20456 is expressed in multiple tissues
- The strongest expression is in PBL, Liver and Spleen

Figure 6

TRAC1 is Predominantly Expressed in Resting T lymphocytes

Figure 7

FLJ20456 Sequence is Most Similar to Two Sequences: Znf313 and STRIN

Ring domain

```
Consensus #1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . F . C . VC . EV . . . PV . . . . C . HVFC . . C . . . . . . . . . .

FLJ20456.pep  MGSVLSTDSGKSAPASATARALERRRDPELFVTSFDCAVCLEVIHQPVB-TRCGHVFCRSCIAISLKN  67
znf313.pep    ----MAAQQRDCGGLAAQLAGPAAEADHLGRFYCPVCLEVYEKPVQ-PCGHVFCSACLQECLKP    59
STRIN.pep     ----MAEDLSAAISYTEDDFYCQEVIKTPVRTTACQHVFCRKCFLTAMRE    49

Consensus #1  . . . . . . . . . . . C . . CR . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . R . H . . . C . KY . . Y . . . . .

FLJ20456.pep  NKWTCDYCRAYLPSE----GVPATDVAKRMKSEYKNCAECDTLVCLSEMRAHIRTCQKYIDKYGPLQE  131
znf313.pep    KKPVCGVCRSALAP----GVRAVELERQIEISTETSCHGCRKNFFLSKIRSHVATCSKYQN-YIMEGV  121
STRIN.pep     SGAHCELCRGNVTRRERACPERALDLENIMRKFSGSCRCCAKQIKFYRMRHHYKSCKKKYQDEYGVSSI  117

Consensus #1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . CP . C . . . . . . L . HC . . H . . . .

FLJ20456.pep  LEETAAR-----...---CVCPFCORELYE-DSLLDHCITHRSERR  166
znf313.pep    KATIKDASLQPRNVPNRY------TFPCPYCPEKNFDQEGIVEHCKLFHSTDTK  169
STRIN.pep     VPNFQISQDSVGNSNRSETSTSDNTETYQENTSSGHPIFKCLCQSNETRQRLLDHCNSNHLFQIV  185

Consensus #1  . . . . . . . . . . . V . CP . C . . . . P . . . . P . . . . . N . . . H . . . . . . . . . Y . F . . . . EE . . . . . . . . . s . . . . . . . . . . .

FLJ20456.pep  PVFCPLCRLIPDENPSFSGNLIRHLQVSHTLFYDDFIDFNIIEEALIRRVLDRSLLEYVNHSNTT.    233
znf313.pep    SVVCPICASMPWGDPNYRLANFREHIQRRHRFSYDTFVDYDVDEEDMMNQVLQRSIIDQ.    229
STRIN.pep     PVTCPICVSLPWGDPSQITRNEVSHLNQRRQLEDYGEFVNLQLDEETQYQTAVEESFQVNI.    246
```
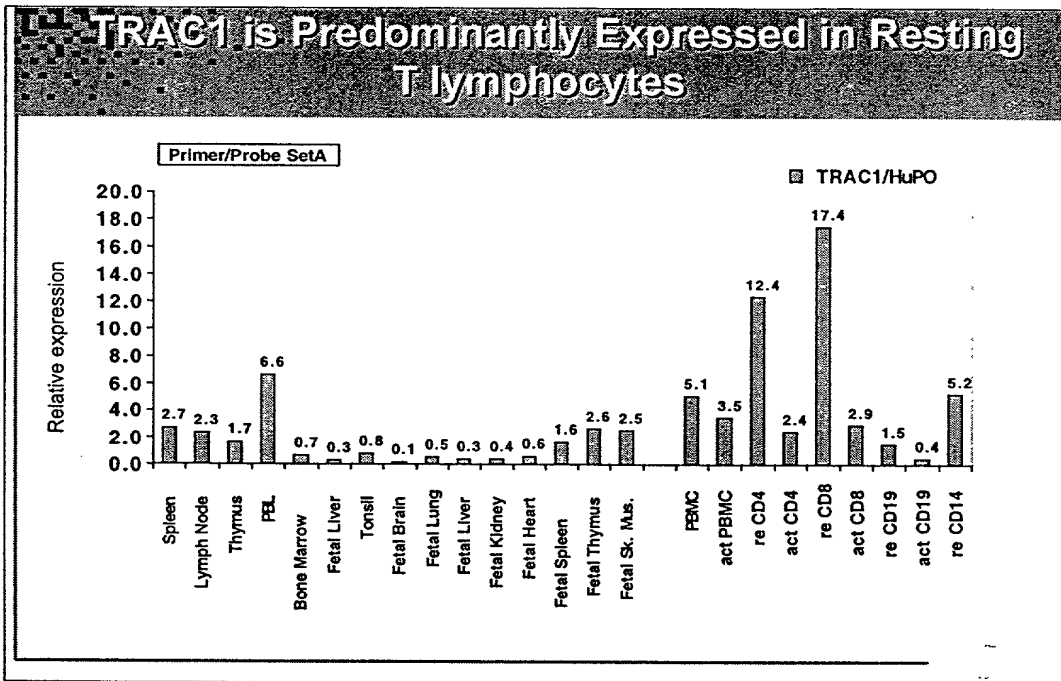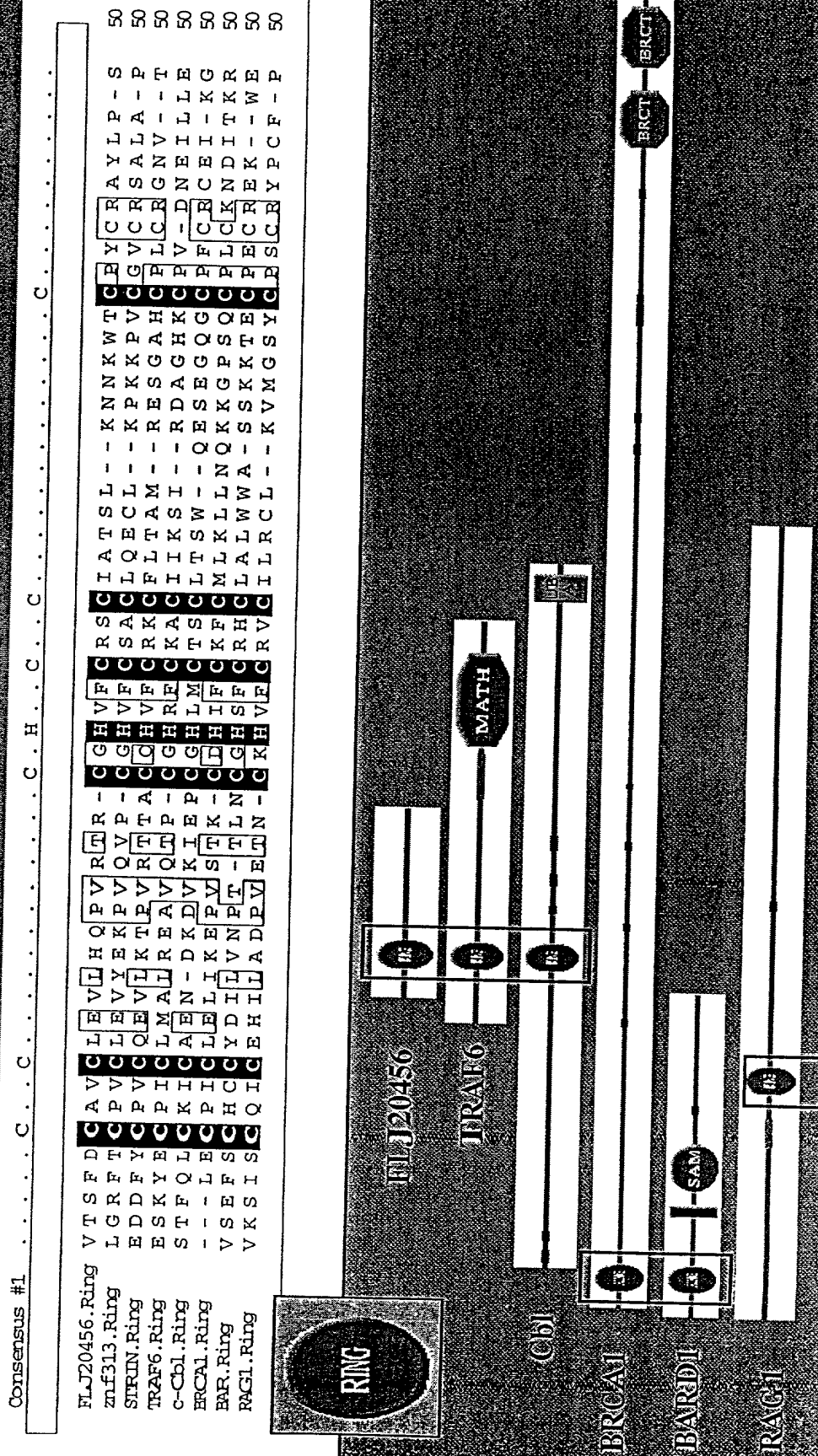
• All three sequences are human
• Murine sequences are not shown

divergence	Percent Identity			
		1	2	3
	1		26.6	22.3
	2	130.4		27.9
	3	140.9	134.7	
		1	2	3

1	FLJ20456.pep
2	znf313.pep
3	STRIN.pep

Figure 8

Alignment of RING Domain Sequences from Various Human Proteins

Consensus #1 . . . . C . . . . C . . C . . . . C . H . . . . . . C . . . . C C

```
                    50
FLJ20456.Ring  V T S F D C A V C L E V I H Q P V R M R - C G H V F C R S C I A T S L - - K N N K W T C B Y C R A Y L P - S   50
znf313.Ring    L G R F T C P V C L E V Y E K P V Q V P - C G H V F C S A C L - - K P K K P V C G V C R S A L A - P   50
SIRIN.Ring     E D D F Y C P V C Q E V L K T P V R T T A - C Q H V F C R K C L T A M - R E S G A H C P L C R G N V - T   50
TRAF6.Ring     E S K Y E C P I C L M A L R E A V Q T P - C G H R F C K A C I I K S I - R D A G H C P V - D N E I L L E   50
c-Cbl.Ring     S T F Q L C K I C A E N - D K D V K I E P - C G H L M C T S C L T S W - I Q E S E G Q G C P F C R C E I - K G   50
BRCA1.Ring     - - L E C P I C L E L I K E P V S T K - C D H I F C K F C M L K L L N Q - K K G P S Q C P L C K N D I T K R   50
BAR.Ring       V S E F S C H C C Y D I L V N P T - T L N - C G H S F C R H C L A L W A - S S K T E C P E C R E K - I W E   50
RAG1.Ring      V K S I S C Q I C E H I L A D P V E T N - C K H V F C R V C I L R C L - - K V M G S Y C R Y P C F - P   50
```

RING

FLJ20456

TRAF6 — MATH — US

Cbl — US

BRCA1 — RING — BRCT BRCT

BARD1 — US — SAM

RAG1 — US

Figure 9

RING finger vs. Zinc finger proteins

Ring-HC: C_3HC_4 = Cys in position 5
Ring H2: C_3H2C_3 = His in position 5

- Ring finger domains have a conserved pattern of Cys and His residues that co-ordinate two zinc atoms to form a cross-brace structure
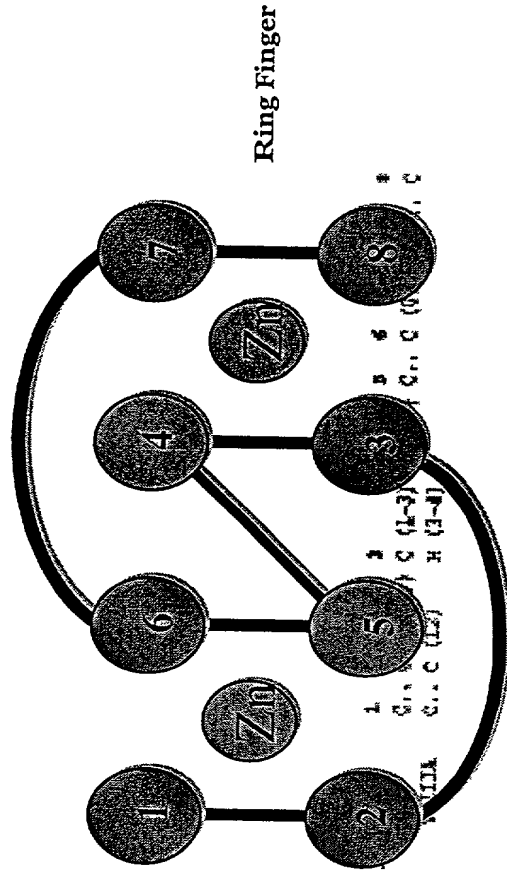
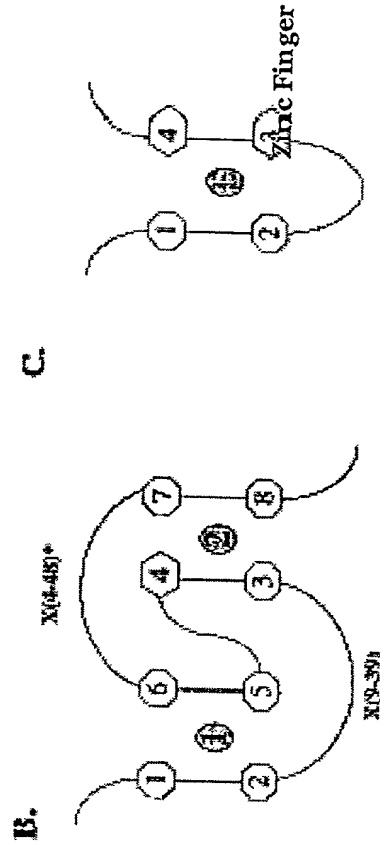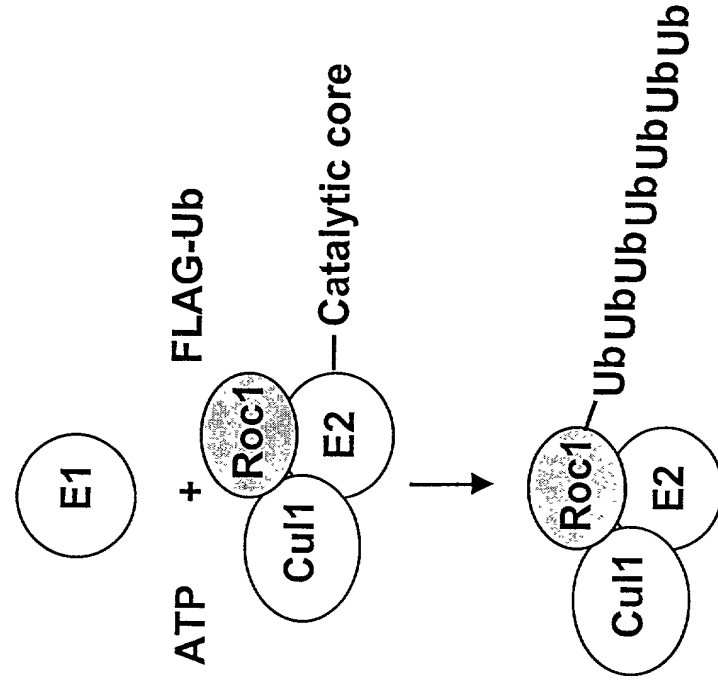- Ring fingers are structurally distinct from zinc fingers

Ring Finger

B.

C.

Zinc Finger

Figure 10

10 A

Ubiquitin Pathway Components

- **E1:** ubiquitin-activating enzyme, with a major isoform that may work broadly
- **E2:** ubiquitin-conjugating enzyme, a class of ~14 enzymes, interacts with E3
- **E3:** ubiquitin ligases, a broad and growing group of activities that promote addition of ubiquitin to specific proteins
- **Proteasome**-a 26S complex containing a 19S lid and base that mediates ATP- and ubiquitin-chain-dependent binding of substrates and a 20S catalytic core with three known proteolytic activities.

10 B

Enzymology of Ubiquitynation

Ub = ubiquitin	E2 = ubiquitin conjugating enzyme
E1 = ubiquitin activating enzyme	E3 = ubiquitin ligase

Figure 11a
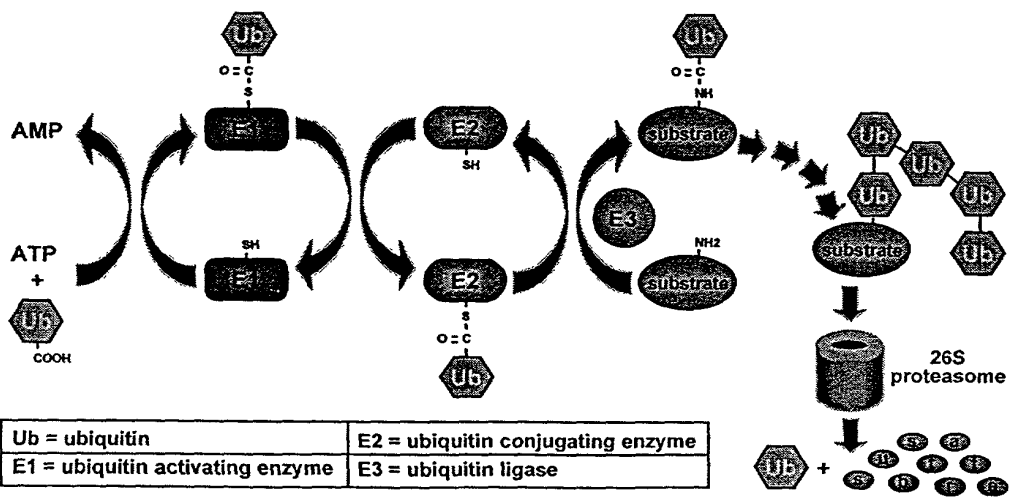
A Reconstituted, Substrate-independent Assay for Studying Ligase Catalysis

Reaction Components

E1:

E2 (UbcH5): GST-fusion (cleaved), E. coli

E3 (Ring/cullin): His-tagged, coexpressed, baculovirus
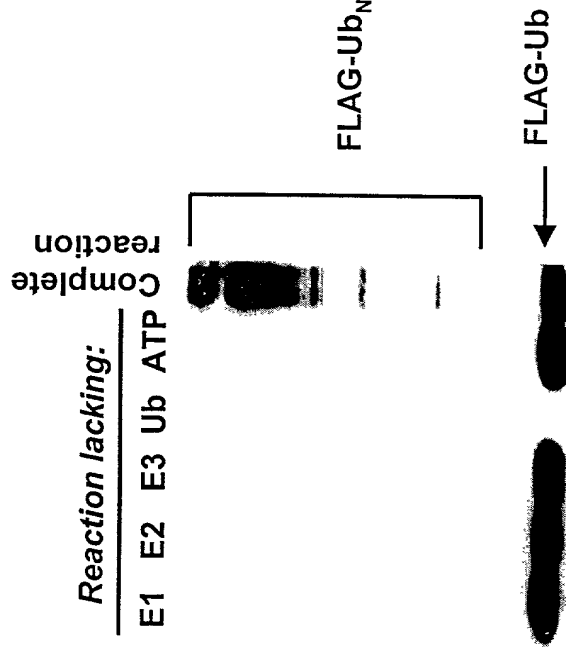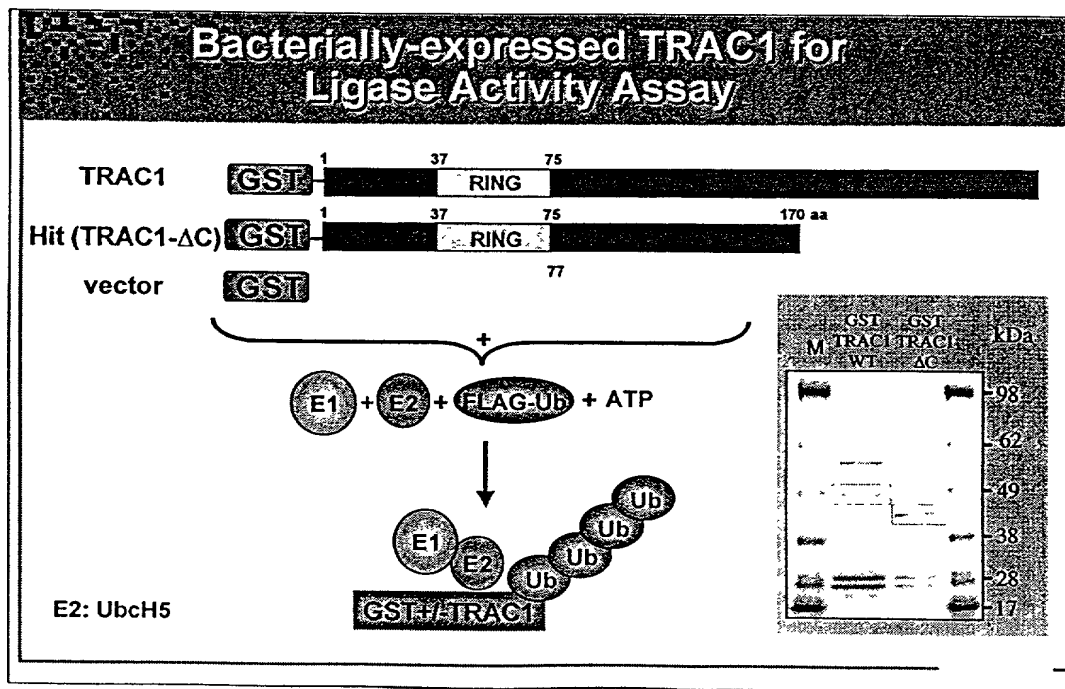
Ubiquitin: FLAG-tagged, E. coli

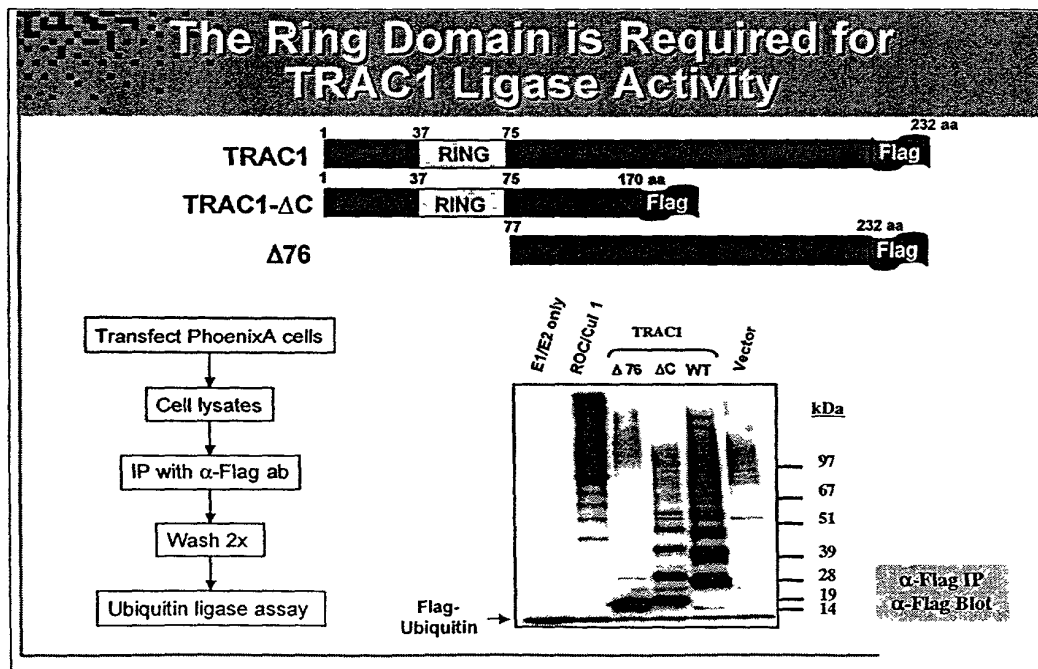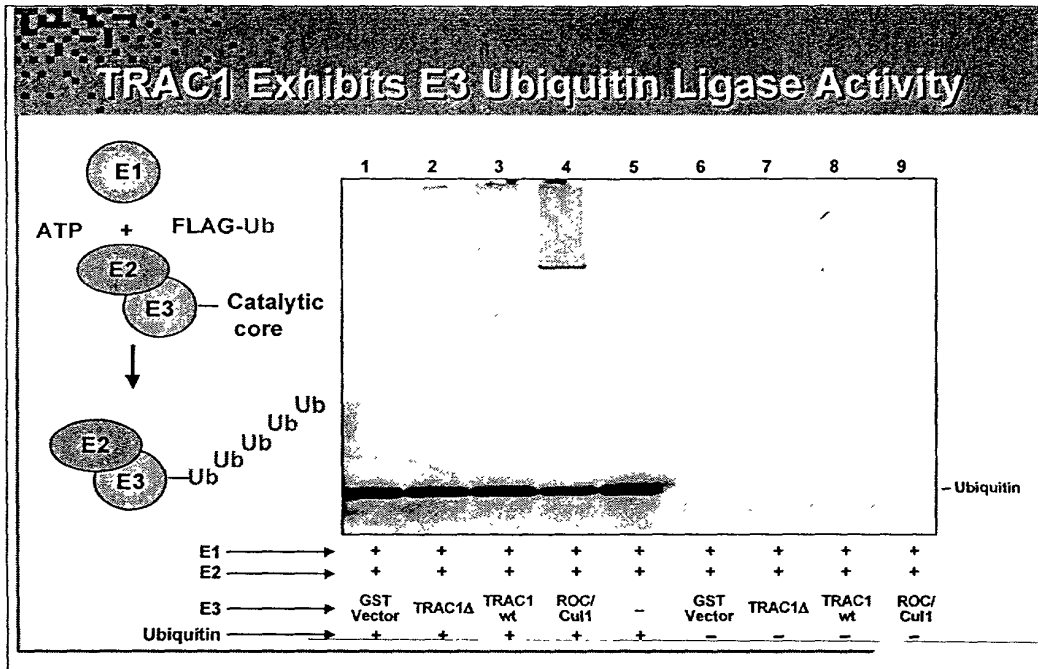The substrate-independent reaction has the same catalytic properties and requirements for Roc1/Cul1 as the substrate-dependent reaction

Figure 11 B

Bacterially-expressed TRAC1 for Ligase Activity Assay

Figure 12

12A

TRAC1 Exhibits E3 Ubiquitin Ligase Activity

	1	2	3	4	5	6	7	8	9
E1	+	+	+	+	+	+	+	+	+
E2	+	+	+	+	+	+	+	+	+
E3	GST Vector	TRAC1Δ	TRAC1 wt	ROC/Cul1	–	GST Vector	TRAC1Δ	TRAC1 wt	ROC/Cul1
Ubiquitin	+	+	+	+	+	–	–	–	–

E1
ATP + FLAG-Ub
E2
E3 — Catalytic core

E2
E3 — Ub Ub Ub Ub Ub Ub

– Ubiquitin

12B

The Ring Domain is Required for TRAC1 Ligase Activity

TRAC1   1 37 75 RING 232 aa Flag
TRAC1-ΔC   1 37 75 RING 170 aa Flag
Δ76   77 232 aa Flag

Transfect PhoenixA cells
↓
Cell lysates
↓
IP with α-Flag ab
↓
Wash 2x
↓
Ubiquitin ligase assay

E1/E2 only   ROC/Cul 1   TRAC1 (Δ76 ΔC WT)   Vector

kDa
97
67
51
39
28
19
14

Flag-Ubiquitin

α-Flag IP
α-Flag Blot

Figure 13

Figure 14

Expression of TRAC-1 mRNA is ~8 fold higher in PBMC than in Jurkat cells
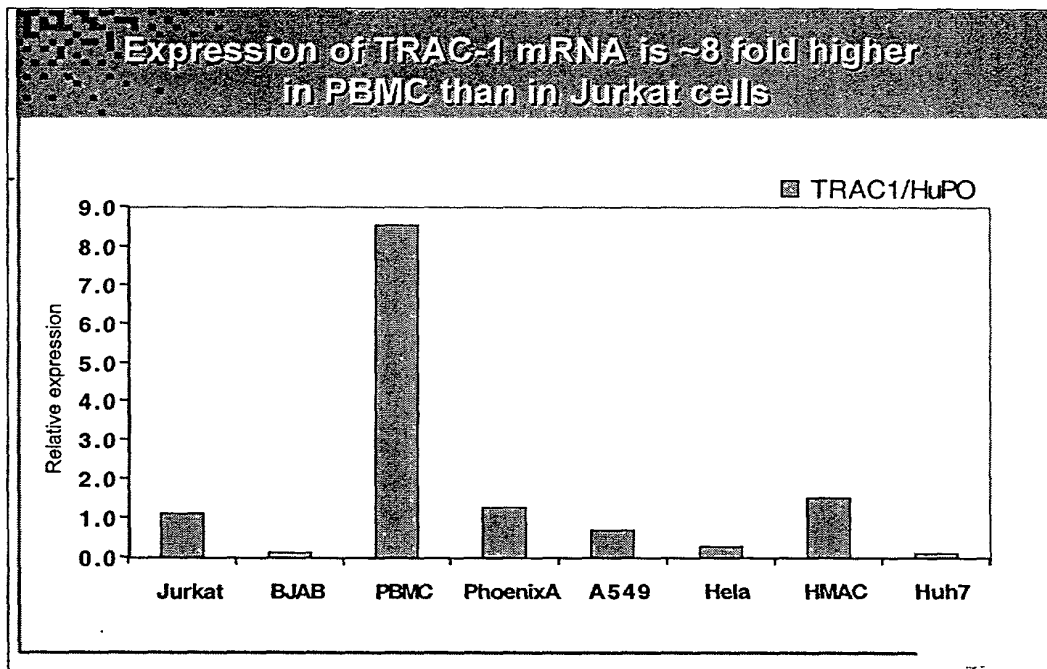
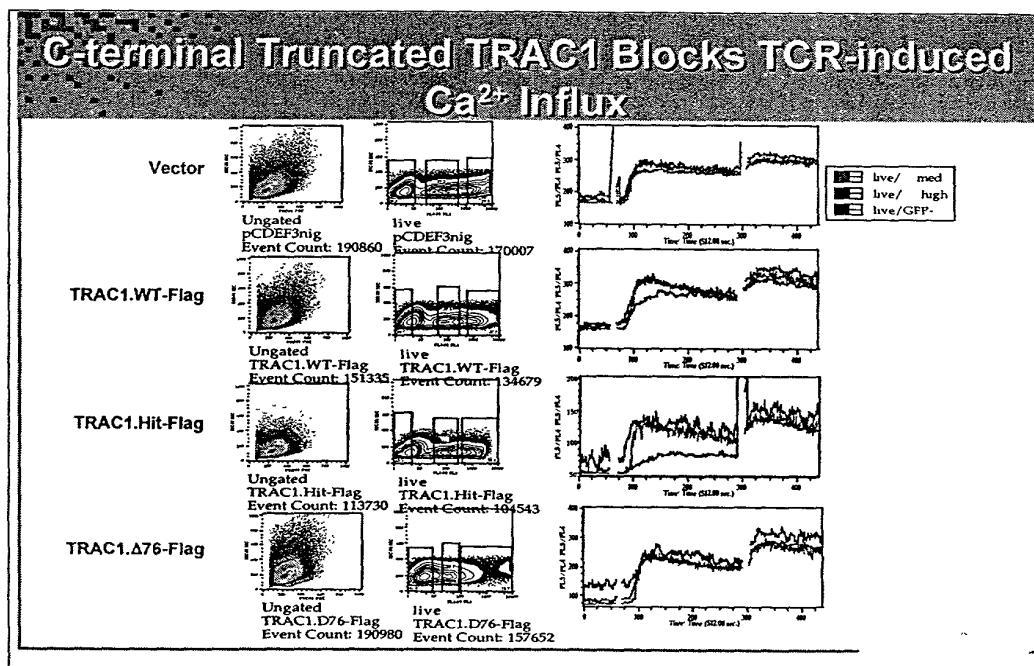Figure 15

C-terminal Truncated TRAC1 Blocks TCR-induced Ca²⁺ Influx

Figure 16

An Intact TRAC1 Ring domain is Required for Inhibition of α-TCR-Induced CD69 Up-regulatio
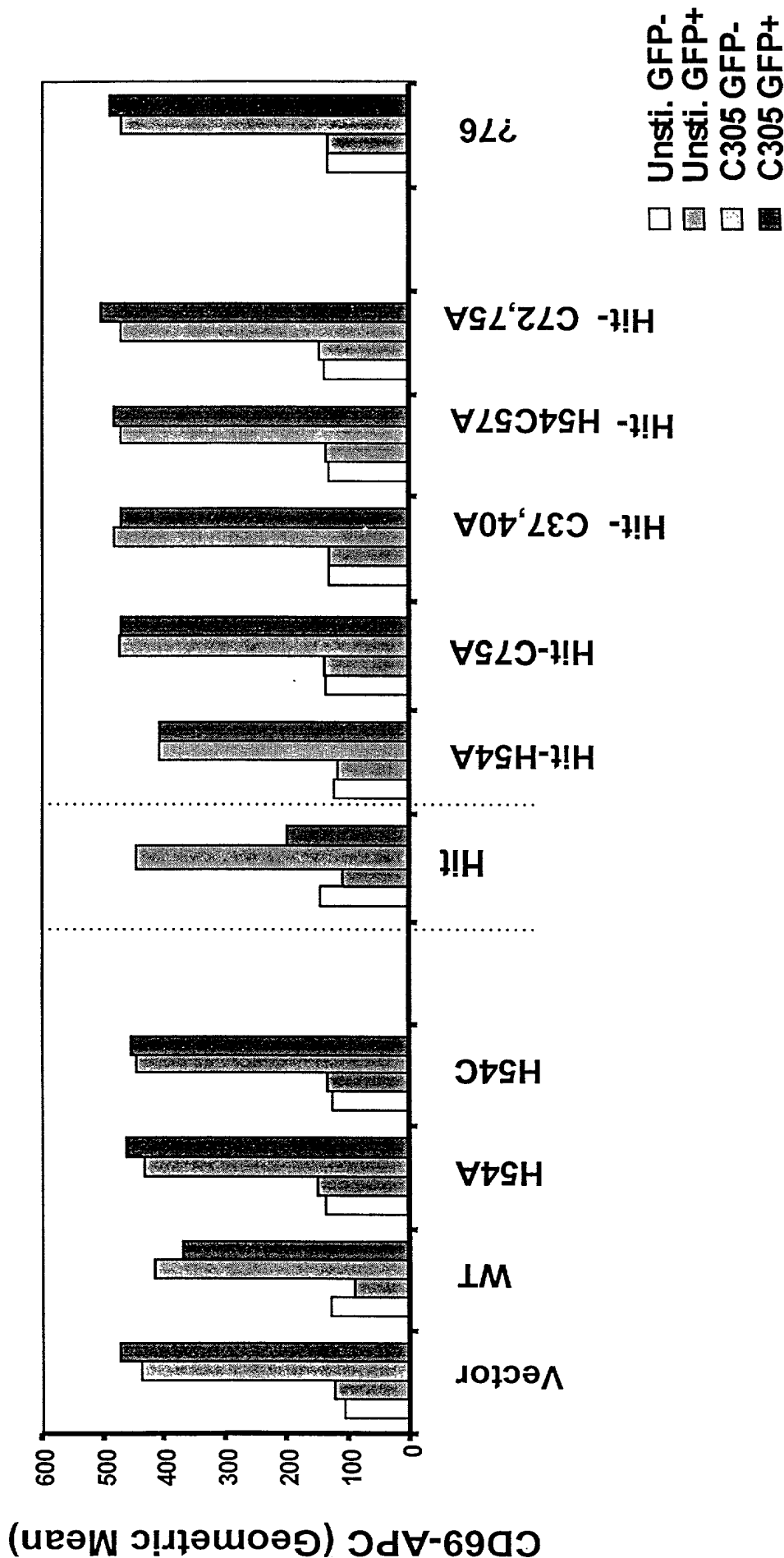
Figure 17

Summary of Functional Effects by Different TRAC-1 constructs

Figure 18

Transiently Transfected TRAC1 Protein Binds to Ubiquitin-Conjugating Enzymes (E2s) UbcH7 and UbcH5 *in vitro*

Cellular Lysate Transfected w/

	pEF vector		pEF.TRAC1.WT

Purified E2s W/ His-tag

10% Lysate — Ni-beads — Ni:UbcH7 — Ni:UbcH5 | 10% Lysate — Ni-beads — Ni:UbcH7 — Ni:UbcH5

← FLAG-TRAC1

kDa
38 —
28 —
17 —
14 —
6 —

Figure 19